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Full Length Research Paper

Physiological and biochemical responses to low temperature stress in hybrid clones of *Populus ussuriensis* Kom. × *P. deltoides* Bartr

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Cuttings of three hybrid clones of *P. ussuriensis* × *P. deltoides* were exposed to different low temperatures (cold and freezing) for 24 h, or consecutive low temperatures (5°C, 0 to 120 h), to determine physiological and biochemical responses to cold stress in these woody plants. Soluble sugar and protein contents increased in the three clones under cold (10, 5°C) stress, malondialdehyde (MDA) concentrations, electrolytes leak and activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) also variously increased at 10, 5, and 0°C. While under lower temperatures (-5, -10, and -15°C) treatment, levels of all except electrolytes leak and MDA gradually decreased relatively. During consecutive low temperature (5°C) treatment, concentrations of soluble protein and MDA, electrolytes leak and activities of CAT, APX and GR increased immediately or after 3 h, however, soluble sugar concentrations and SOD and POD activities slightly decreased at the beginning of the treatment; subsequently increased quickly, and then declined. When exposed to different (10, 5, 0, -5, and -10°C) and consecutive (5°C, after 12 or 24h) low temperature stresses, there were significant differences between the three clones. Levels of soluble sugar and soluble protein, and SOD, CAT, POD, APX and GR activities were relatively higher in clone UD183 than in UD189 or UD191. Levels of electrolyte leak and MDA were lower than in UD189 or UD191. Poplar hybrid clones thus employ various physiological and biochemical responses and mechanisms for coping with low temperature stress. According to the results of this study, we presume that clone UD183 may acquire much more freezing- tolerance than clones UD189 and UD191. Our research will promote understanding of cold acclimation and freezing-tolerance in poplars, and encourage selection of the best clones for the cold regions of northern China.

Key words: Osmotic adjustment, electrolyte leakage, malondialdehyde, antioxidant enzymes, low temperature stress, hybrid clones, populus.

INTRODUCTION

Among abiotic stresses, low temperature (cold and freezing) constitutes one of the major hazards to crops and trees, and limits the survival , productivity and geographical distribution of plants across large areas of the world (Boyer, 1982; Karimzadeh et al., 2006). Low temperatures reduce enzymatic activity, alter metabolism and decrease the photosynthetic capacity of plant tissues (Dubey, 1997). However, many temperate plants can increase their freezing tolerance during exposure to low, but non-freezing temperatures; a process known as cold acclimation (Thomashow, 1999). And physiological and biochemical changes occurring in plants when cooled to freezing temperatures are of great interest in order to

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understand the mechanism of plant adaptation to low temperature.

Traditionally, plant screening for cold and freezing tolerance is based on visual observations in the field. Unfortunately, this screening is subjective and subordinate to other influential factors such as wind, air humidity, exposure, and water status and health conditions of plants that could distort the final result (Mancuso, 2000). Recently, interesting results to quantify freezing tolerance have come from the study of some compatible solutes and antioxidant enzymes, and evaluate membrane damage (Sakai and Larcher, 1987; Bridger et al., 1994; Bettaieb et al., 2007).

It is well known that low temperature stress induces the generation of reactive oxygen species (ROS), such as superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxide radicals (OH-) and singlet oxygen ($1O_2$). These are highly reactive and can seriously impair the normal function of plant cells by lipid peroxidation, protein degradation, and DNA nicking, and may even cause cell death (Chaitanya et al., 2002). In order to keep ROS levels under control, plants possess antioxidative systems composed of enzymatic scavengers, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR), and also some non-enzymatic antioxidants, such as ascorbic acid, α-tocopherols, phenolic compounds and reduced glutathione (Asada, 1992; Bettaieb et al., 2007; Garbero et al., 2011). These biochemical compounds have been found in almost all cellular compartments; demonstrating the importance of ROS detoxification for cell survival (Mittler et al., 2004). In addition, osmotic adjustment provides a potentially important mechanism for freezing tolerance and cold acclimation. This can be achieved via the accumulation of compatible solutes, such as soluble sugar and soluble protein, in protoplasm (Misra and Gupta, 2005). It has been confirmed that soluble sugar and soluble protein play an important role based on their detection in various species of land plants undergoing seasonal cold acclimation (Sakai and Larcher, 1987; Rutten and Santarius, 1992). It has been suggested that they not only serve as osmo-protectants, but also, through their interaction with the lipid bilayer, play a role in protecting cellular membranes from damage caused by dehydration and freezing (Shalaev and Steponkus, 2001; Lee et al., 2002; Karimzadeh et al., 2006).

Poplar (*Populus* spp.), are fast-growing forest trees, widely used for timber, pulp, and paper, and providing potential sources of biomass energy (Perry et al., 2001). Hybrid vigor has been observed in many plants including poplars, and is utilized in poplar breeding to maximize yield, improve wood quality, and enhance resistance to abiotic stresses (including cold, drought, and salinity) and to pests and diseases (Muhle, 1970; Marron et al., 2006; Courty et al., 2010). We used three hybrid clones of *P. ussuriensis* × *P. deltoides* to test physiological and biochemical responses to low temperature stress. Our objective was to investigate the different protective roles of compatible solutes (soluble sugar and soluble protein), and antioxidant enzymes (SOD, CAT, POD, APX and GR), against cold damage under different low temperature (cold and freezing) conditions, and consecutive low temperature treatment; and to detect variation among clones. This research will help understanding of the mechanisms of freezing tolerance and cold acclimation in hybrid clones of *P. ussuriensis* × *P. deltoids*, and to select the best clones for planting in the cold, high latitude regions of northern China.

MATERIALS AND METHODS

Plant materials and experimental design

Cuttings of one year old vegetation were selected from three hybrid clones of *P. ussuriensis* × *P. deltoides* (UD183, UD189 and UD191), in an experimental stand established in PingQuan County (41° 00′ 7″ N, 118° 42′ 17″ E), Hebei Province, northern China, in 2006. The cuttings were planted in basins (15 cm diameter × 10 cm depth) that contained sand, organic matter and vermiculite (3:1:1,v/v/v) in the garden of Beijing Forestry University in March 2010. After sprouting and growth for about 2 months, healthy cuttings with approximately the same crown size and equal height were chosen and moved into growth chambers at the laboratory of Beijing Forestry University; and grown from 1st June to 15th July, 2010. Light intensity was 4,800 lx provided by cool white fluorescent lamps over a 16 h photoperiod. Day and night temperatures were 25±1 and 20±1°C, respectively, and relative humidity was 55 to 85%.

For low temperature treatment, two procedures were used:

1) 135 cuttings from three hybrid clones (45 cuttings from each clone) were treated for 24 h at different temperatures. The nine temperature levels were 25, 20, 15, 10, 5, 0, -5, -10 and -15°C, with 25°C as a control.
2) 135 cuttings from three hybrid clones (45 cuttings from each clone) were exposed to 5°C for different time periods. The eight time periods were 3, 6, 12, 24, 48, 72, 96 and 120 h, with 0 h as a control.

In both 1 and 2, five cuttings of each clone were used for each level or period, and three independent replicates were conducted. Finally, fifteen cuttings of each clone for each level or period were harvested, and the fully expanded leaves that were third to fifth from the apex were collected for investigation of physiological and biochemical traits.

Determining soluble sugar and soluble protein concentrations

Soluble sugar was measured as described by Mohsenzadeh et al. (2006), and concentration was expressed as mg g^{-1} DW (DW is the dry weight after over-drying samples at 85°C for 24 h). Soluble protein was measured as described by Bradford (1976) using bovine serum albumin as a standard, and concentration was expressed as mg g^{-1} DW.

Assessment of electrolyte leakage and malondialdehyde

Electrolyte leakage (EL) was measured using an electrical conductivity meter as described by Lutts et al. (1996). Leaves were excised and washed with deionized water. After drying with filter

paper, 1 g fresh weight of leaves were cut into small pieces (about 1 cm^2) and then immersed in 20 mL deionized water and incubated at 25°C. After 24 h, electrical conductivity (EC1) of the bathing solution was recorded. Those samples were then autoclaved at 120°C for 20 min to completely kill the tissues and release all electrolytes. Samples were then cooled to 25°C and the final electrical conductivity (EC2) was measured. The electrolyte leakage (EL) was expressed following the formula EL = EC1 / EC2 × 100.

Malondialdehyde (MDA) concentration was measured by the thiobarbituric acid (TBA) method described by Xu et al. (2006) with modifications as follows. Fresh leaves (0.3 g) were homogenized in 5 ml 10% (v/v) trichloroacetic acid (TCA) solution and the homogenate was centrifuged at 12,000 g for 15 min. 2 ml of supernatant was removed, added to 2 ml of 0.6% TBA solution, heated in a boiling water bath for 30 min and cooled quickly in an ice bath. The mixture was centrifuged at 12,000 g for 15 min and the resulting supernatant was measured at 532, 600 nm with UV-VIS spectrophotometer (Beckman M36, USA). MDA concentration was calculated and expressed as μmol g^{-1} DW.

Assays of antioxidant defense systems

Fresh leaves (0.5 g) were ground in liquid nitrogen using a mortar and pestle, and ground samples were homogenized in an ice bath, individually, in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12,000 g for 20 min at 4°C, and then the supernatant was used for the following enzyme assays. SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), as described by Giannopolitis and Ries (1977). The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 0.1 ml of enzyme extract. Reaction mixtures were illuminated for 15 min at a light intensity of 75 μmol m^{-2} s^{-1}. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT as monitored at 560 nm. Total SOD activity was expressed as units mg^{-1} protein. CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.0) and 0.1 mM EDTA. This reaction mixture and 750 mM H_2O_2 were heated to 25°C. Then 50 μl of enzyme solution was mixed with 2.9 ml of reaction mixture, and 50 μl 750 mM H_2O_2 was added to begin the reaction. The absorbance at 240 nm was read every 30 s. CAT activity is followed by a decrease in absorbance between 0.5 and 3 min (Aebi, 1984). CAT activity was calculated and expressed as nmol H_2O_2 mg^{-1} protein min^{-1}. POD (EC 1.11.1.7) activity was measured using a modification of the method described by Chance and Maehly (1955). The assay mixture contained 50 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, 10 mM guaiacol and 5 mM H_2O_2. Firstly, 50 μl enzyme solutions was added to the reaction mixture to a total volume of 3.0 ml. Changes in absorbance of the brown guaiacol at 470 nm, between 0.5 and 3.5 min, were recorded to calculate POD activity. This was expressed as μmol guaiacol mg^{-1} protein min^{-1}. APX (EC 1.11.1.11) activity was analyzed by following the decrease in A_{290} (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 1 min, in 3 ml of a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM sodium ascorbate, 2.5 mM H_2O_2, and 200 μl of enzyme extract (Nakano and Asada, 1981). Enzyme activity was expressed as μmol AsA mg^{-1} protein min^{-1}.

A modification of the method used by Halliwell and Foyer (1978) was employed for the assay of GR (EC 1.6.4.2) activity. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA and 5 mM $MgCl_2$. The reaction mixture, 10 mM NADPH, and 10 mM glutathione disulfide (GSSG), were heated to 25°C. Then 50 μl of enzyme solution and 20 μl of 10 mM NADPH were added to the 2.88 ml reaction mixture, and finally 50 μl of 10 mM GSSG was added. GR activity was calculated and expressed as nmol NADH mg^{-1} protein min^{-1}. Soluble protein concentration was determined as described by Bradford (1976), using bovine serum albumin as a calibration standard.

Statistical analyses

For all recorded variables, analyses of variance (ANOVA) were conducted to test differences. Statistical analyses using Duncan's method were performed using SAS 8.2 for Windows (*SAS* Institute Inc. USA).

RESULTS

Soluble sugar and soluble protein

Soluble sugar and protein concentrations within the leaves of hybrid clones of *P. ussuriensis* × *P. deltoides* changed mildly at 25°C (control) and 20°C, for 24 h (Figures 1a and 2a), and slight differences between clones were exhibited. However, soluble sugar and protein concentrations increased when clones were exposed to 15, 10 and 5°C, and which increased sharply at 10 and 5°C especially, but decreased gradually when the temperature decreased to 0, -5, -10 and -15°C. Significant differences among the three clones were detected; soluble sugar and protein concentrations within clone UD183 were significantly higher than those within UD189 or UD191 under different low temperatures stress expect soluble protein concentrations within the three clones at -15°C.

During consecutive low temperatures (5°C) stress, soluble sugar concentrations within the leaves of the three hybrid clones decreased from 0 to 3 h (Figure 1b), but increased sharply from 6 to 24 h, subsequently increased slowly from 24 to 72 or 96 h, and then declined quickly. Soluble protein concentrations within the three clones gradually increased between 0 and 72 h, but decreased rapidly from 72 to 120 h (Figure 2b). In addition, soluble sugar and protein concentrations within clone UD183 were significantly higher than those within the other two clones from 3 to 120 h.

Electrolyte leakage (EL) and malondialdehyde (MDA)

Electrolyte leakage and MDA concentrations within the leaves of the three hybrid clones significantly increased when they were exposed to different low temperatures (10, 5, 0, -5, -10, and -15°C) stress (Figures 3a and 4a). And those within clone UD183 were obviously lower than those within UD189 and UD191 at different low temperatures, but at 25°C (control) and 20°C, EL and MDA within UD183 were higher than in UD189 and UD191.

During consecutive low temperature treatment, EL and MDA within clone UD183 increased slowly from 0 to 48 h,

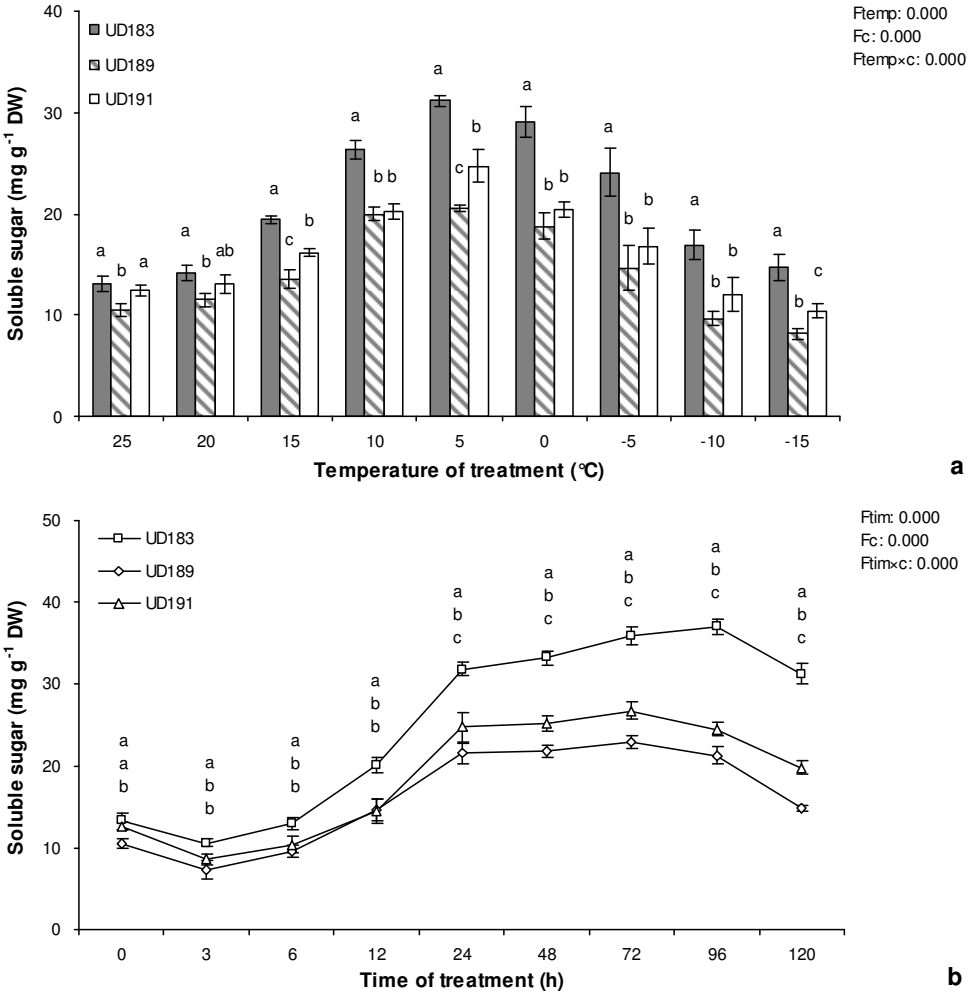

Figure 1. Concentrations of soluble sugar in cuttings of three poplar hybrid clones exposed to (a), different temperatures for 24 h; and (b), low temperatures (5℃) for different periods. The data presented are means ± SE (n = 5). Lowercase letters refer to differences among the three hybrid clones at the same temperature (a) or time treatment (b). Values followed by letters are significantly different from each other at $P<0.05$ according to Duncan's method. F temprature, the effect of the different temperature treatment; Fc, the effect of clones; Ftim, the effect of low temperature treatment for different periods; Ftemp×c, the temperature × clones interaction effect; Ftim×c, the time × clones interaction effect, as determined by ANOVA.

but increased sharply from 48 to 72 or 96 h, then increased slowly (Figures 3b and 4b); while increase trends of those within UD189 and UD191 were greater than UD183 between 0 and 48 h. Interestingly, at 0 h of 5℃ treatment (control), EL and MDA within clone UD183 were higher than those within UD189 and UD191; but from 6 to 120 h, EL and MDA within clone UD183 were significantly lower than those within the other two clones.

Antioxidant defense systems

Under different temperatures' treatment, antioxidant enzymes including SOD, CAT, POD, APX and GR within the leaves of the three hybrid clones changed mildly at 25℃ (control), 20 and 15℃, for 24 h (Figures 5a, 6a, 7a, 8a and 9a); CAT, APX and GR activities within the three clones and SOD activities within clone UD183 increased gradually when they were exposed to 10, 5 and 0℃, but decreased when the temperature decreased to -5, -10 and -15℃ (Figures 5a, 6a, 8a and 9a); however, POD activities within the three clones and SOD activities within clone UD189 and UD191 increased gradually when they were exposed to 10 and 5℃, but decreased when the temperature decreased to 0, -5, -10 and -15℃ (Figures 5a and 7a). In addition, SOD activities within clone UD183 were significantly higher than those within UD189 and UD191 under different temperatures stress; CAT and

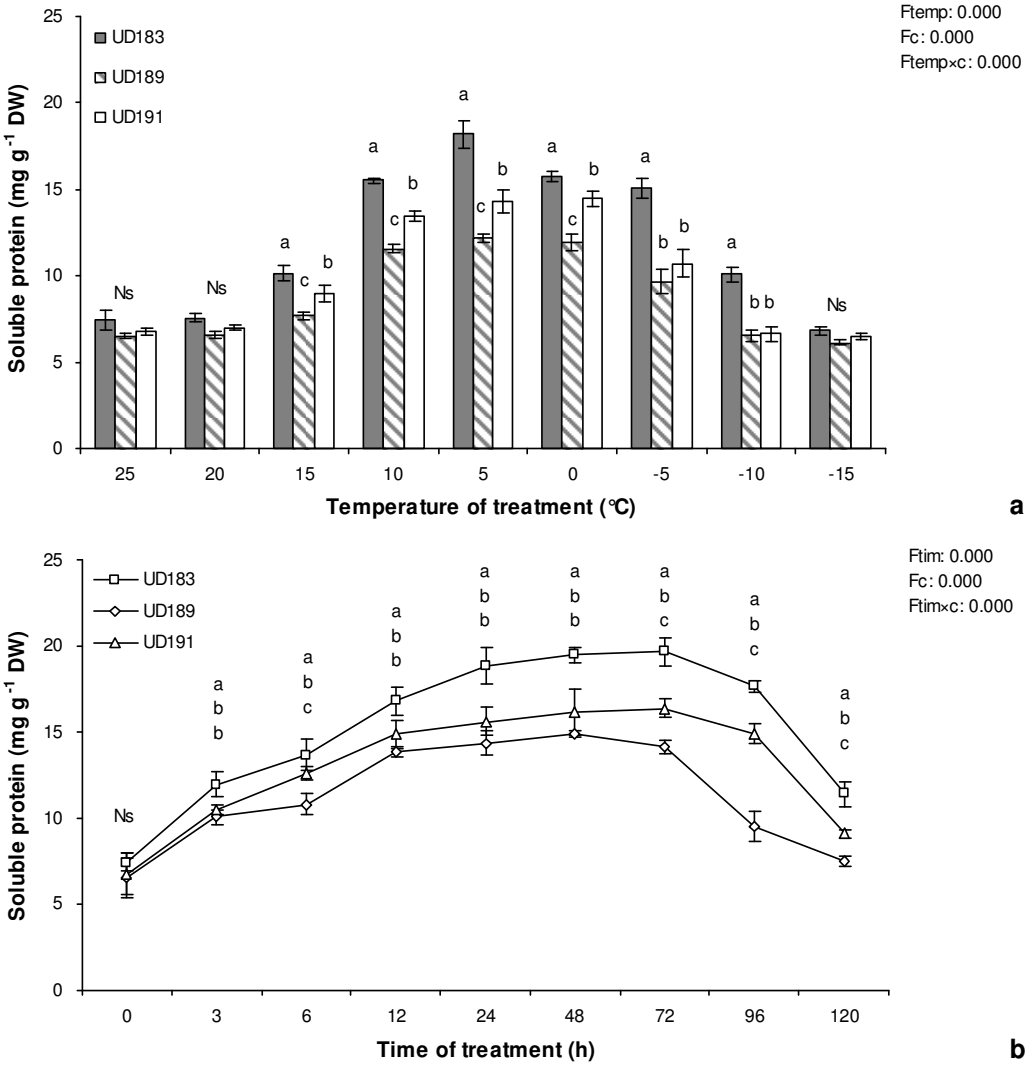

Figure 2. Concentrations of soluble protein in the cuttings of three poplar hybrid clones exposed to (a), different temperatures for 24 h; and (b), low temperatures (5℃) for different periods. The data presented are means ± SE (n = 5). Lowercase letters refer to differences between the three hybrids clones at the same temperature (a), or time treatment (b). Values followed by letters are significantly different from each other at $P<0.05$ according to Duncan's method. F temperature, the effect of the different temperature treatment; Fc, the effect of clones; Ftim, the effect of low temperature treatment for different periods; F temperature ×c, the temperature × clones interaction effect; Ftim×c, the time × clones interaction effect, as determined by ANOVA.

POD activities within clone UD183 were significantly higher than those within UD189 and UD191 at 10, 5, 0, -5 and -10℃; APX activities within clone UD183 were significantly higher than those within UD189 and UD191 at 10, 5, 0, -5, -10 and -15℃; GR activities within clone UD183 were significantly higher than those within UD189 and UD191 at 20, 10, 5, 0, -5 and -10℃.

During consecutive low temperatures (5℃) stress, SOD and POD activities within the three hybrid clones decreased from 0 to 3 or 6 h, subsequently increased sharply, and then declined (Figures 5b and 7b); and SOD activities within clone UD183 were significantly higher than those within UD189 and UD191 all the time, POD activities within clone UD183 were significantly higher than within UD189 and UD191 from 6 to 120 h. CAT and APX activities within the three hybrid clones increased all along (Figures 6b and 8b); moreover, CAT activities within clone UD183 were significantly higher than those within UD189 and UD191 at 6 h and from 24 to 120 h, APX activities within clone UD183 were significantly higher than those within UD189 and UD191 between 24 and 120 h. While GR activities within the three hybrid clones decreased slightly at the beginning of stress, then gradually increased from 3 to 120 h (Figure 9b); in addition, GR activities within clone UD183 were significantly higher than those within UD189 and UD191

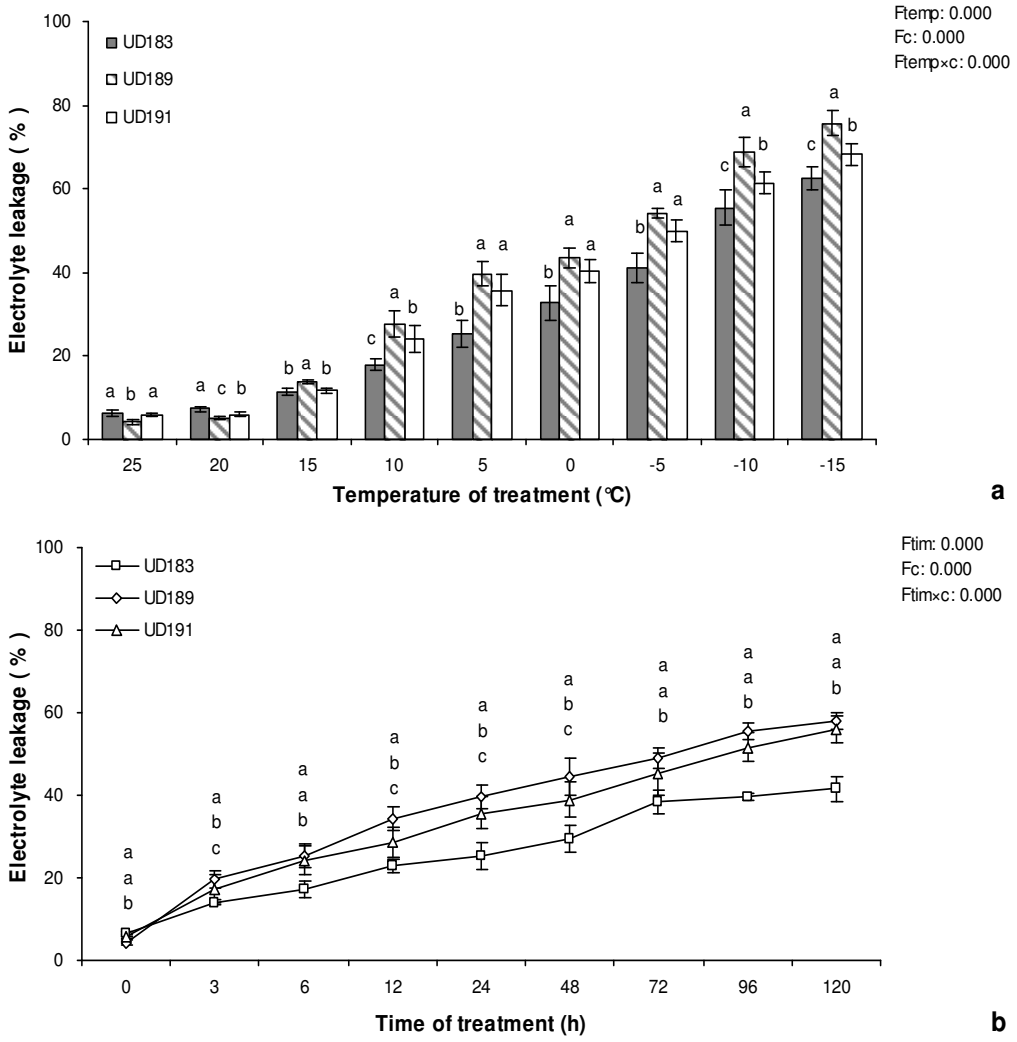

Figure 3. Electrolyte leakage (EL) in the cuttings of three poplar hybrid clones exposed to (a), different temperatures for 24 h; and (b), low temperatures (5℃) for different periods. The data presented are means ± SE (n = 5). Lowercase letters refer to differences between the three hybrid clones at the same temperature (a), or time treatment (b). Values followed by letters are significantly different from each other at $P<0.05$ according to Duncan's method. Ftemp, the effect of the different temperature treatment; Fc, the effect of clones; Ftim, the effect of low temperature treatment for different periods; Ftemp×c, the temperature × clones interaction effect; Ftim×c, the time × clones interaction effect, as determined by ANOVA.

at 0 h (control) and from 24 to 120 h.

DISCUSSION

It is known that carbohydrates such as soluble sugars accumulate as a response to low temperature stress. They can play multiple roles, including cryo- and osmo-protection, and energy provision for general metabolism and for synthesis of new stress-responsive compounds (Leslie et al., 1995; Ferullo and Griffith, 2001). For example, during cold acclimation, soluble sugars accumulate in preventing a putative freezing/thawing cycle (Stushnoff et al., 1998); it was also observed that leaves of these plants such as wheat (*Triticum aestivum* L.), *Arabidopsis* (*Arabidopsis thaliana* L.), and poplar, accumulated high levels of soluble sugar under cold treatment (Savitch et al., 1997; Strand et al., 1999; Renaut et al., 2005). We found that soluble sugar concentrations in the leaves of three hybrid poplar clones increased under cold stress, which is consistent with the above studies. However, soluble sugar concentrations decreased gradually when our three poplar clones were exposed to lower temperatures (0, -5, -10, and -15℃), and also decreased suddenly after long periods at low temperature (5℃, 96 to 120 h). These findings coincide

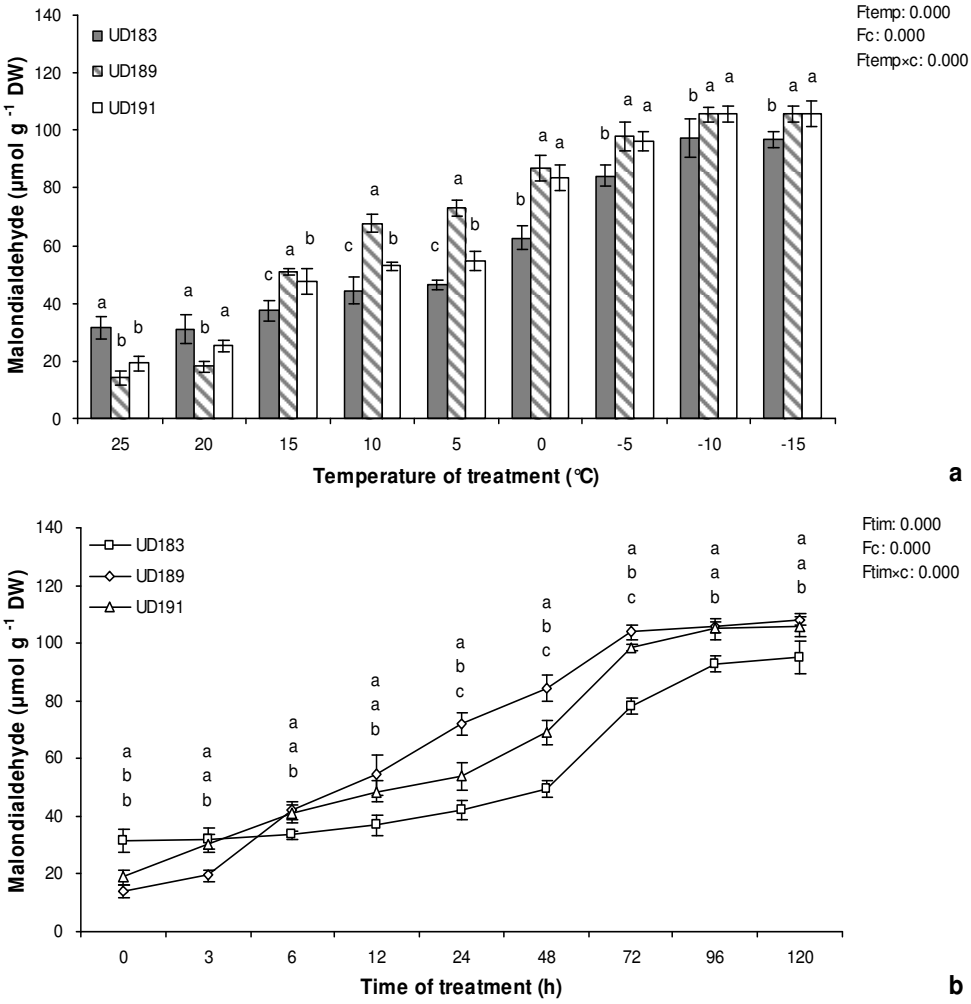

Figure 4. Concentrations of malondialdehyde (MDA) in the cuttings of three poplar hybrid clones exposed to (a), different temperatures for 24 h; and (b), low temperatures (5℃) for different periods. The data presented are means ± SE (n = 5). Lowercase letters refer to differences between the three hybrid clones at the same temperature (a), or time treatment (b). Values followed by letters are significantly different from each other at $P<0.05$ according to Duncan's method. Ftemp, the effect of the different temperature treatment; Fc, the effect of clones; Ftim, the effect of low temperature treatment for different periods; Ftemp×c, the temperature × clones interaction effect; Ftim×c, the time × clones interaction effect, as determined by ANOVA.

with the results of Li et al. (2008). Possible explanations include plants being unable to acclimate to sudden that plants expended rather than accumulated sugar.

Alterations in the protein content of plant tissues have long been associated with cold acclimation and freezing tolerance. Briggs and Siminovitch (1949) observed an accumulation of water-soluble protein during the winter season when cold hardiness was maximal. Many others have recorded similar increases in the protein content of higher plants (Yoshida, 1984; Crosatti et al., 1996; Renaut et al., 2005). In our studies, the finding that a rapid and stable change in the protein synthesis occurred in poplar leaves upon exposure to different low temperatures (10, 5, 0, and -5℃) and short term of low freezing; after suffering a long period of cold stress, synthetic and metabolic activities were affected, meaning temperature (5℃, 0 to 72 h) was consistent with the hypothesis put forth by Weiser (1970) that cold acclimation, leading to greater freezing tolerance. However, proteins synthesized at low temperature that function to increase freezing tolerance, would gradually disappear along with freezing tolerance (Guy and Haskell, 1987). This phenomena was also detected in this studies for long term of exposure to low temperature (5℃, 72 to 120 h).

It is generally considered that plasma membrane has a primary role in determining the cold and freezing injury of plant cells (Steponkus and Wiest, 1979; Yoshida and

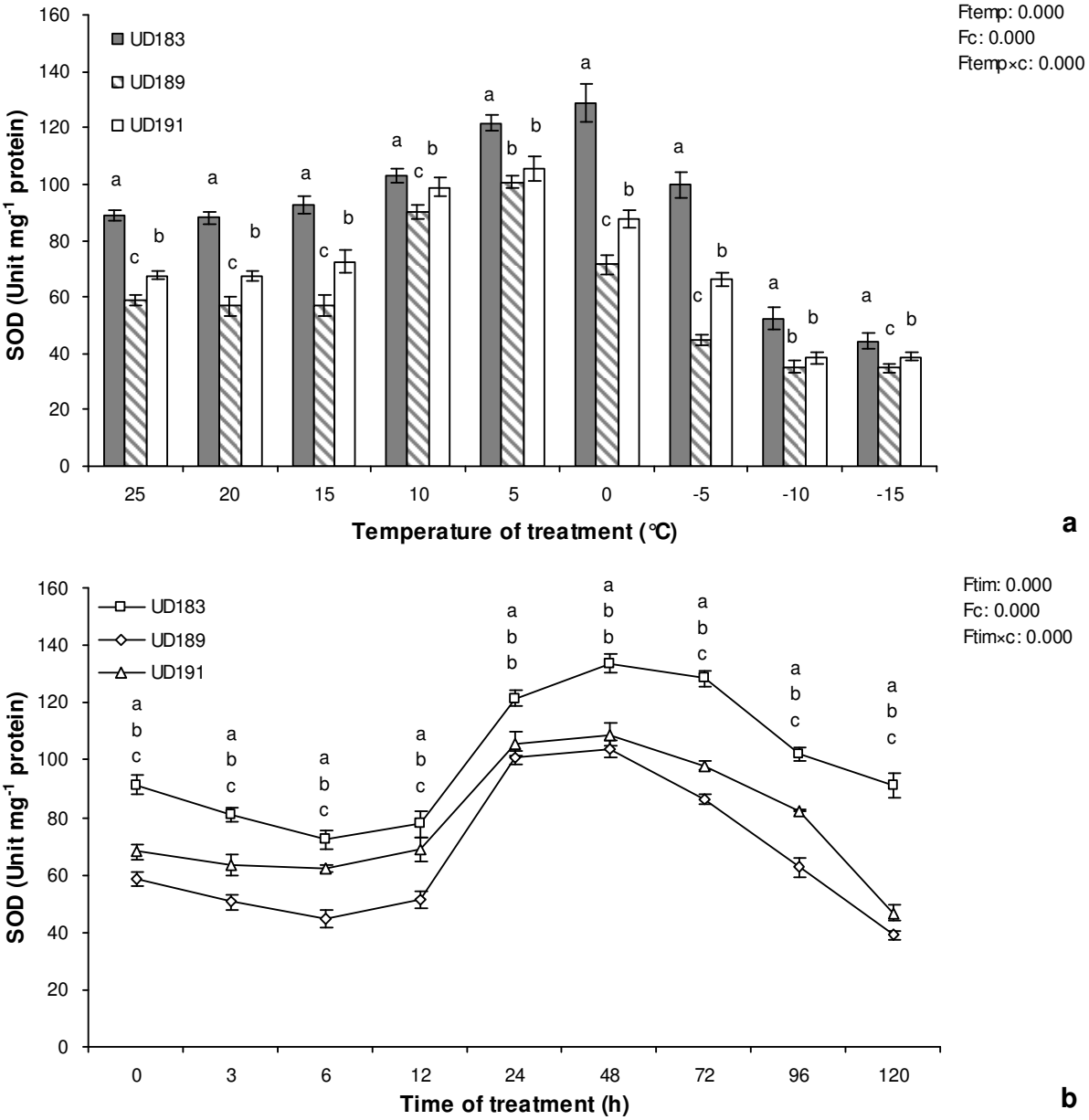

Figure 5. Activities of superoxide (SOD) in the cuttings of three poplar hybrid clones exposed to (a), different temperatures for 24 h; and (b), low temperatures (5℃) for different periods. The data presented are means ± SE (n = 5). Lowercase letters refer to differences between the three hybrid clones at the same temperature (a), or time treatment (b). Values followed by letters are significantly different from each other at $P<0.05$ according to Duncan's method. Ftemp, the effect of the different temperature treatment; Fc, the effect of clones; Ftim, the effect of low temperature treatment for different periods; Ftemp×c, the temperature × clones interaction effect; Ftim×c, the time × clones interaction effect, as determined by ANOVA.

Uemura, 1984). And increased rates of electrolyte leakage occur in a variety of stressed tissues and have been used to evaluate membrane damage following cold and freezing (Wright and Simon, 1973; Simon, 1974). In addition, malondialdehyde (MDA) is one of the final products of stress-induced lipid peroxidation of polyunsaturated fatty acids (Leshem, 1987), and has been considered a maker for cold sensitivity (Jouve et al., 1993; Xu et al., 2006). Under different and consecutive low temperatures treatments of our studies, electrolyte leakage and MDA concentrations in our three hybrid poplar clones increased, especially under lower temperatures (-5, -10, and -15℃) and long term of low temperature (5℃, 48 to 72 or 96 h) stress, the even more increasing was exhibited. This is consistent with research into Manila grass (*Zoysia matrella*) (Wang et al., 2009).

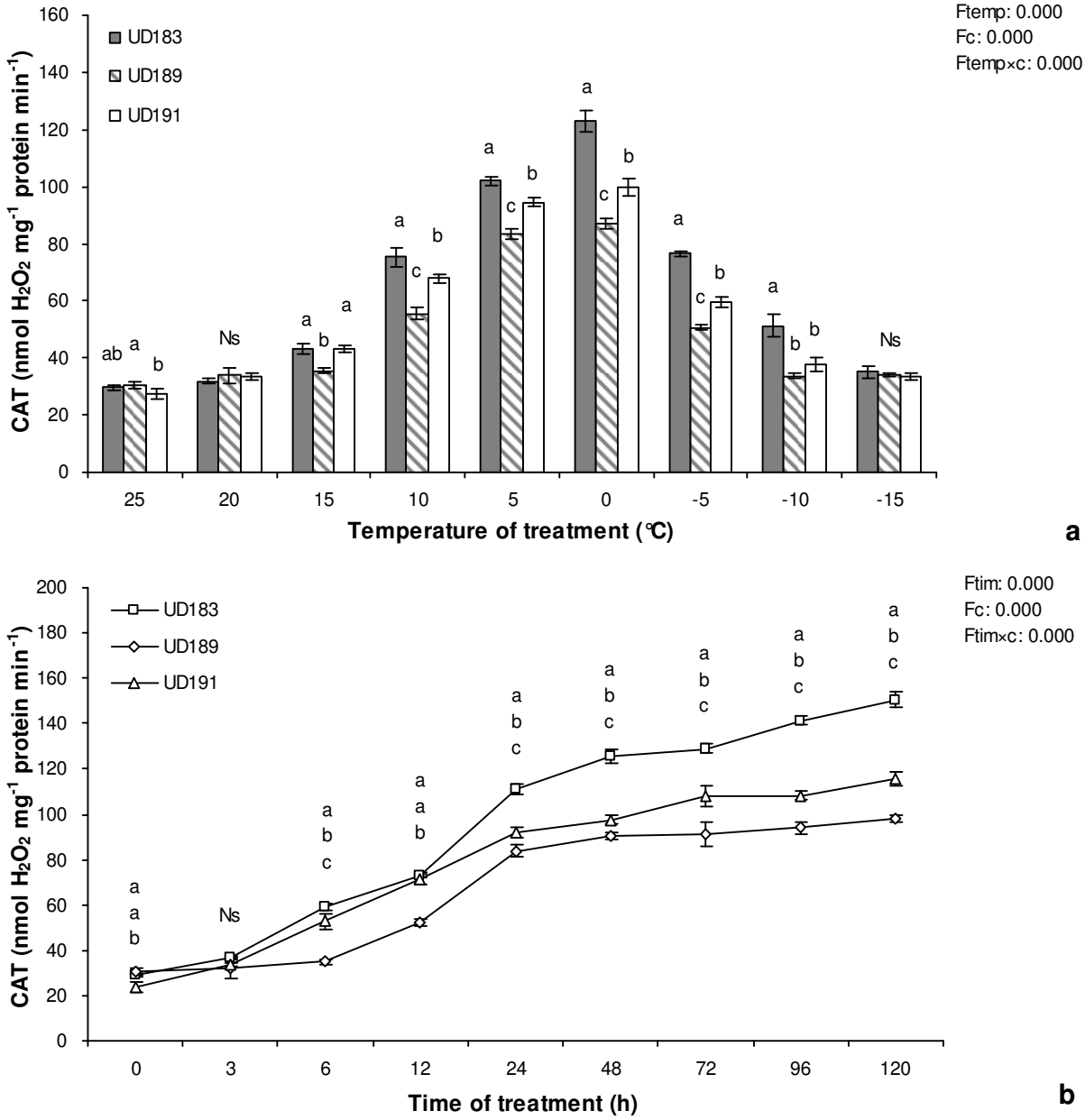

Figure 6. Activities of catalase (CAT) in the cuttings of three poplar hybrid clones exposed to (a), different temperatures for 24 h; and (b), low temperatures (5℃) for different periods. The data presented are means ± SE (n = 5). Lowercase letters refer to differences among the three hybrid clones at the same temperature (a), or time treatment (b). Values followed by letters are significantly different from each other at $P<0.05$ according to Duncan's method. Ftemp, the effect of the different temperature treatment; Fc, the effect of clones; Ftim, the effect of low temperature treatment for different periods; Ftemp×c, the temperature × clones interaction effect; Ftim×c, the time × clones interaction effect, as determined by ANOVA

As a consequence of temperature lowering or low temperature prolonging, membrane lipids commonly undergo phase transition, that is, liquid-crystalin or fluid to gel or solid, which temporarily affect membrane permeability during transient periods of temperature decrease (Simon, 1974; Leshem, 1992). Irreversible permeability changes may occur when certain lipids aggregate to form an inverted structure with hexagonal packing symmetry, which disrupts the membrane bilayer causing an increased permeability of plasma membrane to water and solutes (Uemura et al., 1995; Xin and Browse, 2000).

Moreover, low temperature stress causes physiological disorders within plants and induces production of ROS (Mittler, 2002). Accumulation of ROS breaks the balance between ROS production and the capacity of plants to

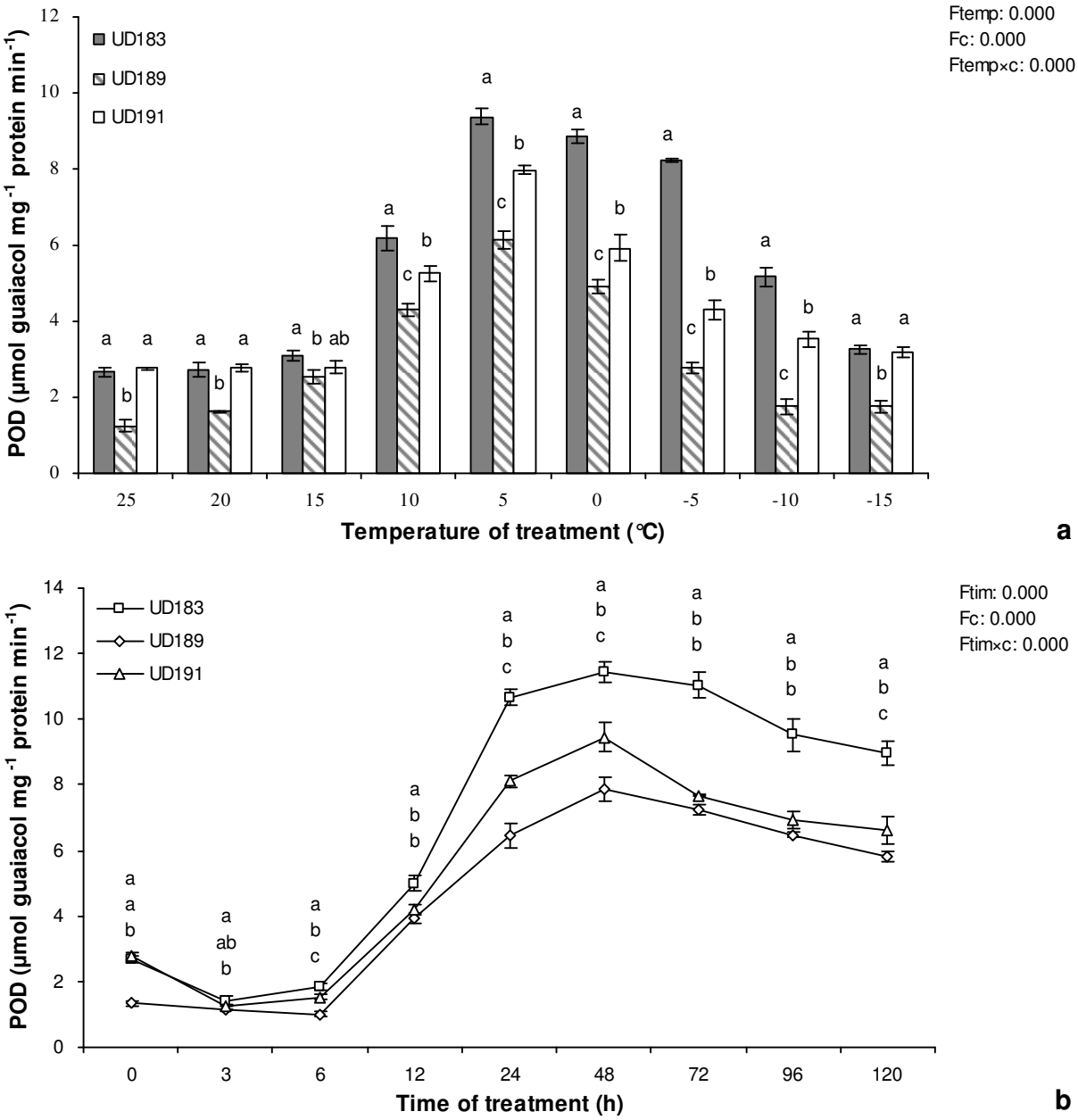

Figure 7. Activities of peroxidase (POD) in the cuttings of three poplar hybrid clones exposed to (a), different temperatures for 24 h; and (b), low temperatures (5℃) for different periods. The data presented are means ± SE (n = 5). Lowercase letters refer to differences between the three hybrid clones at the same temperature (a), or time treatment (b). Values followed by letters are significantly different from each other at $P<0.05$ according to Duncan's method. F temperature, the effect of the different temperature treatment; Fc, the effect of clones; Ftim, the effect of low temperature treatment for different periods; F temperature ×c, the temperature × clones interaction effect; F tim ×c, the time × clones interaction effect, as determined by ANOVA.

scavenge for these; thus inducing destructive oxidation processes, including membrane lipid peroxidation and protein oxidation (Herbinger et al., 2002). Therefore, it is vital for plants to adjust enzymatic and non-enzymatic antioxidant mechanisms to control the ROS over-production and protect their cells (Foyer et al., 1994a, b). Higher plants possess efficient antioxidant enzymes including SOD, CAT, POD, APX and GR (Jaleel et al., 2009; Abdel and He, 2011). And improvement in antioxidant enzyme activity has been considered to be related to cold acclimation and freezing tolerance in plants (Sala, 1998). Within a cell, SOD constitutes the first line of defense against ROS. We observed an increase in the activity of SOD in poplar hybrid clones when exposed to different low temperatures (10, 5, 0℃). However, during consecutive low temperature (5℃)

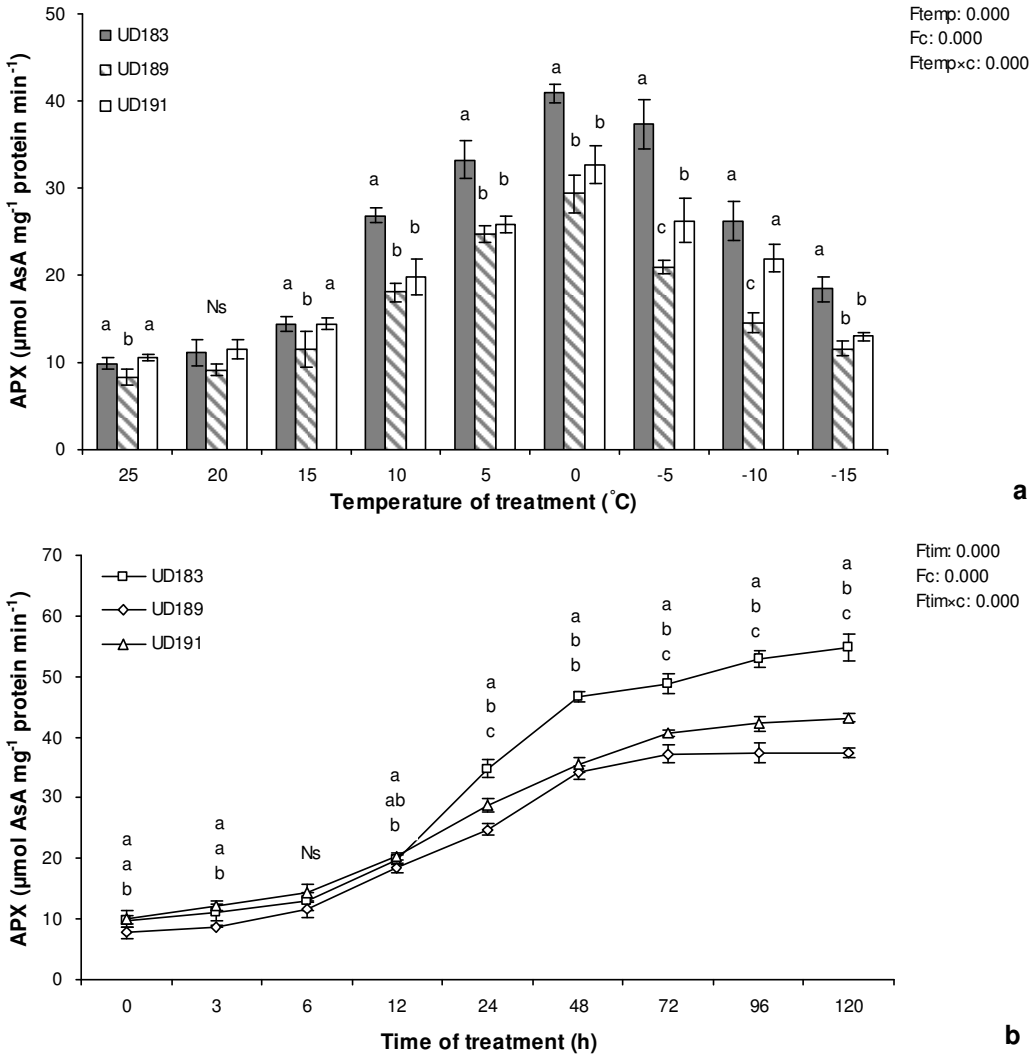

Figure 8. Activities of ascorbate peroxidase (APX) in the cuttings of three poplar hybrid clones exposed to (a), different temperatures for 24 h; and (b), low temperatures (5℃) for different periods. The data presented are means ± SE (n = 5). Lowercase letters refer to differences among the three hybrid clones at the same temperature (a), or time treatment (b). Values followed by letters are significantly different from each other at $P<0.05$ according to Duncan's method. F temperature, the effect of the different temperature treatment; Fc, the effect of clones; Ftim, the effect of low temperature treatment for different periods; F temperature×c, the temperature × clones interaction effect; Ftim×c, the time × clones interaction effect, as determined by ANOVA.

treatment, SOD activity declined under sudden cold stress, then sharply increased, and finally decreased. These changes in SOD activity suggested that our hybrid poplar clones were sensitive at the beginning of low temperature stress, and then had enough SOD activity to catalyze the dismutation of O_2^- to O_2 and H_2O_2; but that subsequently SOD activity declined with lower temperatures and consecutive low temperatures, This appears consistent with research into Crofton weed (*Eupatorium adenophorum*) (Li et al., 2008). CAT and POD are chloroplastic or cytosolic enzymes that appear to play an essential protective role in scavenging processes when coordinated with SOD activity (Massacci et al., 1995). Some researchers support the theory that elevated POD activity scavenges H_2O_2 (Jung, 2004). We observed an increase in the activities of CAT and POD in our three hybrid clones under different low temperatures (10, 5, 0, and -5℃) and consecutive low temperatures (5℃, 3 to 120 h for CAT, 3 to 48 h for POD). This appears consistent with previous studies of Manila grass (*Zoysia matrella*) (Wang et al., 2009) under cold stress. CAT and POD activities decreased with lower temperatures, but levels remained higher than in controls. It is obvious that CAT and POD cooperating with SOD play an important role in preventing plants from suffering the adverse effects of cold and freezing. APX is considered another

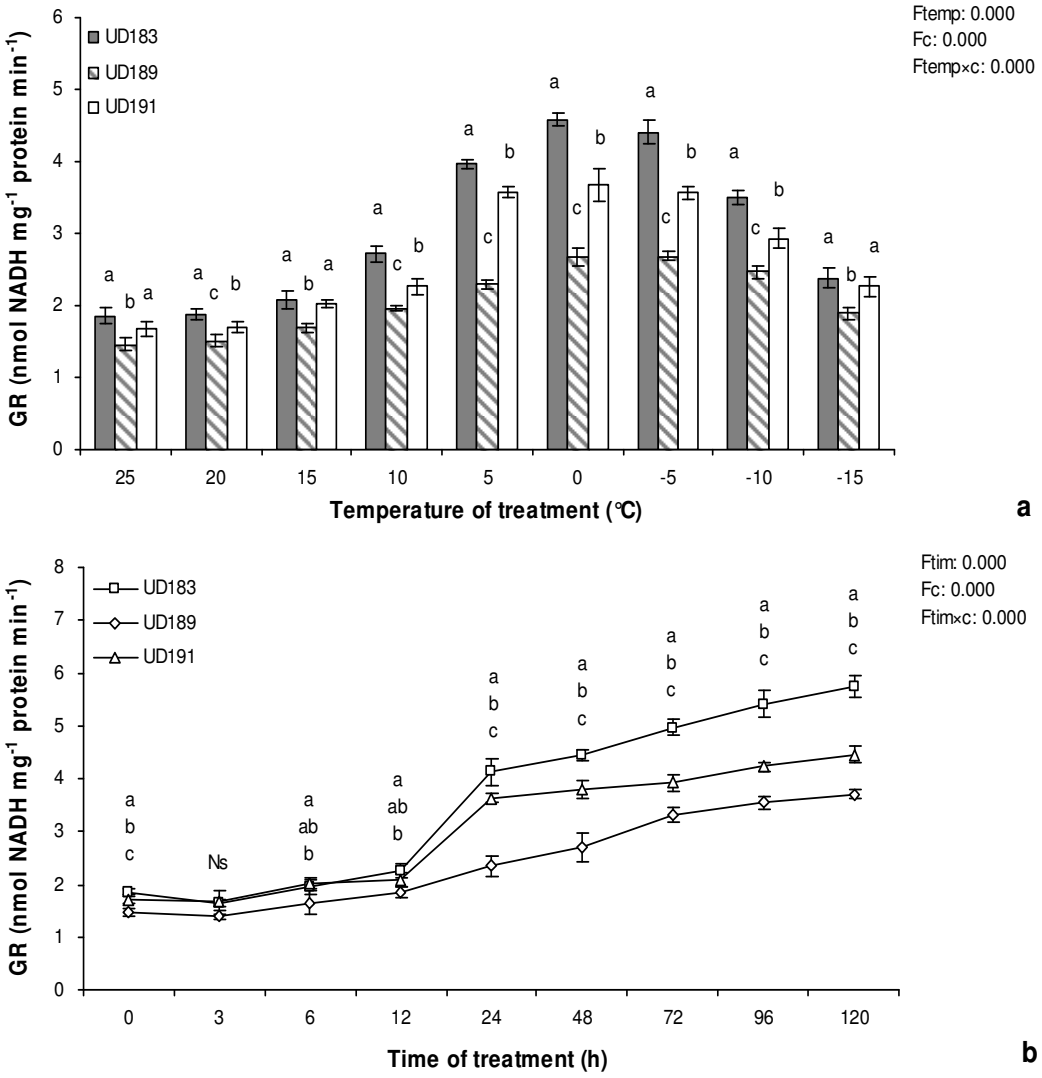

Figure 9. Activities of glutathione reductase (GR) in the cuttings of three poplar hybrid clones exposed to (a), different temperatures for 24 h; and (b), low temperatures (5℃) for different periods. The data presented are means ± SE (n = 5). Lowercase letters refer to differences among the three hybrid clones at the same temperature (a), or time treatment (b). Values followed by different letters are significantly different from each other at $P<0.05$ according to Duncan's method. F temperature, the effect of the different temperature treatment; Fc, the effect of clones; Ftim, the effect of low temperature treatment for different periods; F temperature × c, the temperature × clones interaction effect; Ftim×c, the time × clones interaction effect, as determined by ANOVA.

important H_2O_2-scavenging enzyme in plant cells during environmental stress (Contour et al., 2006), and GR is known to be involved in the regeneration of ascorbate (Foyer and Halliwell, 1976; Horemans et al., 2000). APX and GR activities in our hybrid poplar clones were relatively higher under freezing temperatures (0, -5℃) and during long exposure to low temperatures (5℃, 24 to 120 h). The dramatic increase in APX and GR activity might be because of the localization of APX in cytosol, and subsequently higher affinity for H_2O_2 (Asada, 1992), and, in addition, the activation of GR to regulate the oxidation/reduction status of GSH after it has been changed by low temperatures. Some authors (Kocsy et al., 2001) stressed the important role of GR in the low-temperature tolerance of maize plants, whereas others working with the chilling of tolerant wheat plants (Janda et al., 2003) did not find any significant changes in GR activity even after severe freezing (-15℃).

In our studies, for physiological and biochemical response to low temperature stress, there were significant differences among the three poplar hybrid clones (Figures 1 to 9). All these results showed clone UD183 accumulated much more soluble sugar and protein contents, SOD, CAT, POD, APX and GR activities

than UD189 and UD191, to cope with low temperature stress, and the cell membrane of UD183 suffered the least oxidative damage. According to these results, we presumed that clone UD183 may acquire much more freezing-tolerance than UD189 and UD191 when they were under the low temperature stress. The significant differences also detected among the different temperature treatment and low temperature treatment for different periods (Figures 1 to 9), which suggested that different low temperature stress induced distinct physiological and chemical responses.

In conclusion, different and consecutive low temperatures stress causes cell membrane damage to poplars. However, poplars can employ a broad spectrum of physiological and biochemical mechanisms to cope with these stresses. Different low temperatures (cold and freezing) induce distinct physiological and chemical responses, and consecutive low temperatures also produce these changes. Our three poplar hybrid clones displayed significant differences in physiological and biochemical response to low temperature stresses; we presumed that clone UD183 may acquire greater freeing-tolerance than UD189 and UD191. Our results form a basis for the further study of the physiological, cytological and molecular mechanisms of cold acclimation and freezing-tolerance, and provide some evidence for the need to select suitable poplar genotypes for cold environments.

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Abbreviations:

APX, Ascorbate peroxidase; **CAT,** catalase; **EC,** electrical conductivity; **EDTA,** ethylenediamine-N,N,N',N' tetraacetic acid; **EL,** electrolyte leakage; **FW,** fresh weight; **GR,** glutathione reductase; **GSSG,** glutathione disulfide; **H_2O_2,** hydrogen peroxide; **MDA,** malondialdehyde; **NADH,** nicotinamide adenine dinucleotide diaphorase; **NBT,** nitroblue tetrazolium; **POD,** peroxidase; **PVP,** polyvinylpyrrolidone; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase; **TBA,** thiobarbituric acid; **TCA,** trichloroacetic acid.

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